Federal Republic of Germany DE 203 15 159 U1 2/26/2004
German Patent and Trademark Office

Utility Model

Application date: 10/1/2003 IPC[7]: C07H 21/00

Registration date: 1/22/2004

Published in the Patentblatt [Patent Journal]: 2/26/2004

Union priority: 60/467,117 4/30/2003 US Name and residence of the holder: Artus Gesellschaft für molekularbiologische Diagnostik und Entwicklung mhB, 22767 Hamburg, DE	Name and residence of the representative: Uexküll & Stolberg, 22607 Hamburg

The following data was taken from documents filed by the Applicant

Title: A Kit for Detecting a Novel Coronavirus Associated with the Severe Acute Respiratory Syndrome (Severe Acute Respiratory Syndrome, SARS)

[Claim 1]

Figure

Key:

Top left box: [top word illegible]

Elution volume

gereinigte Probe = purified specimen

glaskapillare = glass capillary

Specification

[1001] The SARS-associated virus, that is provisionally designated as HPAC (Human Pneumonia-Associated Coronavirus), was recently identified by Drosten et al. and a qualitative real-time RT-PCR (Reverse Transcriptase Polymerase Chain Reaction) was disclosed (Drosten et al. (2003) "Identification of a novel coronavirus in Patients with Severe Acute Respiratory Syndrome", published in the New England Journal of Medicine at www.nejm.org,April 10, 2003).

[0002] Based on this sequence data, an efficient, sensitive and reliable quantitative real-time RT-PCR method was developed.

Abstract of the invention

[0003] The present invention relates to an efficient, sensitive and reliable quantitative real-time RT-PCR method for detecting the Severe Acute Respiratory-Syndrome-associated virus (SARS-associated virus) as well as oligonucleotides and kits for detecting the SARS-associated virus.

Description of the figures

[0004] Figure 1: Addition of the internal control to the purification method. Schematic work flow for the control of purification methods and PCR inhibition.

- It should be ensured that the solutions are completely defrosted [thawed], carefully mixed and centrifuged.

[0005] Figure 2: Addition of the internal control to the RealArt™ master. Schematic work flow for the control of the PCR inhibition.

[0006] Figure 3: Reverse transcription of the RNA.

[0007] Figure 4: Initial activation of the hot start enzyme.

[008] Figure 5: Amplification of the cDNA.

[0009] Figure 6: Cooling off.

[0010] Figure 7: Demonstration of the quantification standard (HPA coronavirus LC QS 1 – 4) in a fluorimeter canal F1/F2. NTC: Non-template control

[0011] Figure 8: Demonstration of the internal control (IC) in the fluorimeter canal F3/back-F1 in parallel with simultaneous amplification of quantification standards (HPA coronavirus LC QS 1 – 4) NTC: Non-template control.

Detailed description of the invention

[0012] The invention relates to a method for detecting the virus associated with the severe acute respiratory syndrome (SARS-associated

virus) and designated as HPAC (Human Pneumonia-Associated coronavirus), in which a real-time RT-PCR reaction is carried out using a biological probe. According to an embodiment the forward primer has a length of approximately 18 to 31 nucleotides and bonds to an area defined by nucleotides 69 to 98 of the sequence shown in SEQ ID NO:1, and in which the reverse primer has a length of approximately 18 to 31 nucleotides and bonds to an area defined by nucleotides 123 to 168 of the sequence shown in SEQ ID NO:1, and in which the probe has a length of approximately 18 to 35 nucleotides and bonds to an area defined by nucleotides 89 to 132 of the sequence shown in SEQ 2D NO:1, and in which the probe is marked with two dyes, one of which is a fluorescent reporter dye and one is a quencher dye, and in which at least one dye is a fluorescent dye and in which the SARS virus is detected by a demonstration of the real-time fluorescence when an amplification of virus-specific sequence takes place.

[0013] The forward primer can bond to an area of approximately nucleotide 1 to approximately nucleotide 240 of the SEQ ID NO:1. The reverse primer can bond to an area of approximately nucleotide 60 to approximately nucleotide 300 of the SEQ 2D NO:1. The probe can therefore bond to an area approximately between nucleotide 21 and approximately nucleotide 279 of SEQ ID NO:1. In general, it is useful to obtain an amplification product that is at least an 80mer.

[00 14] According to another embodiment the forward primer has a length of approximately 18 to 35 nucleotides and binds to an area defined by the nucleotides from approximately 1 to approximately 240 of the sequence shown in SEQ ID NO:1; in which [embodiment] the reverse primer has a length of approximately 18 to 35 nucleotides and binds to an area defined by the nucleotides from approximately 60 to approximately 300 of the sequence shown in SEQ 2D NO:1, and in which the probe has a length of approximately 12 to 40 nucleotides and binds to an area defined by the nucleotides from approximately 21 to approximately 279 of the sequence shown in SEQ ID NO:1; and in which the probe is marked with two dyes, one of which is a fluorescent reporter dye and one is a quencher dye and in which at least one dye is a fluorescent dye, and in which the SARS virus is detected by demonstrating the real-time fluorescence when an amplification of virus-specific sequence takes place.

[0015] According to a special embodiment the forward primer has the sequence shown in SEQ ID NO:2, the reverse primer has the sequence shown in SEQ ID NO3 and the probe has the sequence shown in SEQ ID NO:4.

[0016] Especially useful bonding regions within SEQ 2D NO:1 extend from approximately nucleotide 30 to approximately nucleotide 100 (A), from approximately nucleotide 120 to approximately nucleotide 170 (B) and from approximately nucleotide 230 to approximately nucleotide 270 (C). Forward- and reverse primers can thus be selected from each selection

from the areas A, B and C, that is, when forward- and reverse primers bond within areas A and B the probe would have to bond "between them", that is, to the sequence amplified in this manner. The primer combinations A+C or B+C, that determine the sequence area to which the probe the bonds are likewise possible.

[0017] The length of the primers can vary between approximately 18 and approximately 35 nucleotides or assume every specific value within this stated area. According to a particular embodiment the primers have a length between approximately 18 to 31 nucleotides and bond within an area from nucleotide 69 to nucleotide 98 or from nucleotide 123 to nucleotide 168.

[0018] The length of the probe can vary between approximately 12 and 40 nucleotides or assume any specific value within this stated area that is useful for demonstrating the amplified sequence.

[0019] Furthermore, it is possible to carry out the methods using the minor groove (small furrow of the DNA) bonding principle in which the length of the probe can be short up to approximately 12 nucleotides.

[0020] According to another embodiment it is possible to combine two probes (e.g., one probe with a length of approximately 12 nucleotides and one probe with a length of approximately 6 to 7 nucleotides) if the method is carried out in accordance with the LightCycler principle, in which light collector dyes and quencher dyes are used as probes. This method is sufficiently known to the expert in the art. As an alternative thereto, probes in accordance with the quenched FRET principle can be used, that is

disclosed in Krupp et al., "Nucleic Acid Preparations of Pathogens from Biological Samples for Real-Time PCR Analysis", Nuclear Acids Isolation Methods (B. Bowien & P Dürre, editors), American Scientific Publishers, Stevenson Ranch, 2002.

[0021] The following individual primers are specifically excluded from the individual primers of the invention: (1) a primer that bonds to the area from nucleotide 68 to nucleotide 87 (corresponds to BNITMS1 in Drosten et al.); (2) a primer that bonds to the area from nucleotide 82 to nucleotide 101 (corresponds to BNIinS in Drosten et al.); (3) a primer that bonds to the area from nucleotide 34 to nucleotide 57 (corresponds to BNIoutS2 in Drosten et al.); (4) a primer that bonds to the area from nucleotide 124 to nucleotide 145 (corresponds to BNITMAs2 in Drosten et al.); (5) a primer that bonds to the area from nucleotide 169 to nucleotide 190 (corresponds to BNIinAs in Drosten et al.); and (6) a primer that bonds to the area from nucleotide 203 to nucleotide 223 (corresponds to BNIoutAs in Drosten et al.).

[0022] Furthermore, as regards the methods of the invention and the primer pairs of the invention, the specific combinations of (1) and (4), (2) and (5) as well as (3) and (6) of the specifically excluded primers described above are likewise excluded form the primer pairs of the invention and from the methods of the invention when using such primer pairs. However, it should be understood that the individual primers (1) to (6) can be used in the

methods of the invention as part of a primer pair with other primers that do not correspond to primers (1) to (6) if this is desired.

[0023] According to a special embodiment the reporter dye is FAM, 6-FAM, 5-FAM and ALE-XA-288. According to another embodiment the quencher dye is TAMRA, DABCYL or QSY.

[0024] The detection method is either a qualitative or a quantitative method. The detection is in particular the quantitative demonstration of the real-time fluorescence signal intensity.

[0025] The biological probe used to detect the SARS-associated virus is a bodily fluid and in particular sputum, stool or blood.

[0026] The advantage of the present invention resides in the fact that for the first time the quantitative demonstration of SARS-associated virus with a theoretical demonstration boundary of 10 genome equivalents is possible in the PCR, which corresponds to 120 RNA copies per ml of the biological probe. The RT-PCR method in accordance with the present invention is positive in at least 95% of the investigated cases.

[0027] The PCR efficiency corresponds substantially to the theoretical value of 2. The efficiency is in particular at least approximately 1.9.

[0028] It is worthy of mention that the method disclosed by Drosten et al. has a significantly lower efficiency with a demonstration boundary of over 100 genome equivalents in the PCR.

[0029] The present invention therefore makes available for the first time an efficient, sensitive and reliable quantitative real-time RT-PCR method for detecting the virus associated with the Severe Acute Respiratory-Syndrome (SARS-associated virus).

[0030] The present invention also relates to a kit for carrying out the above method for detecting the virus associated with the Severe Acute Respiratory-Syndrome (SARS-associated virus).

[0031] The kit is in particular a kit for detecting the SARS-associated virus by real-time RT-PCR and comprises a forward primer with a length of approximately 18 to 31 nucleotides that bonds to an area defined by nucleotides 69 to 98 of the sequence shown in SEQ 2D NO:1; comprises a reverse primer with a length of approximately 18 to 31 nucleotides that bonds to an area defined by nucleotides 123 to 168 of the sequence shown in SEQ ID NO:1; and comprises a probe with a length of approximately 18 to 35 nucleotides that bonds to an area defined by nucleotides 89 to 132 of the sequence shown in SEQ ID NO:1, which probe is marked with two dyes, one of which is a fluorescent reporter dye and one is a quencher dye, and at least one dye is a fluorescent dye.

[0032] According to a special embodiment the kit comprises a forward primer with the sequence shown in SEQ ID NO:2, a reverse primer with the sequence shown in SEQ ID NO:3 and a probe with the sequence shown in SEQ ID NO:4.

[0033] According to a special embodiment the reporter dye is FAM, 6-FAM, 5-FAM and ALE-XA-288. According to another embodiment the quencher dye is TAMRA, DABCYL or QSY.

[0034] It is sufficiently known to an expert in the art that a kit in accordance with the invention can also comprise enzymes and reagents required for carrying out a real-time RT-PCR reaction.

[0035] The invention also relates to an oligonucleotide with a length of approximately 18 to 31 nucleotides that bonds to an area defined by nucleotides 69 to 98 of the sequence shown in SEQ ID NO:1. The oligonucleotide has in particular the sequence shown in SEQ 1D NO:2. The invention also relates to an oligonucleotide with a length of approximately 18 to 35 nucleotides that bonds to an area defined by nucleotides 1 to 240 of the sequence shown in SEQ ID NO:1.

[0036] The invention also relates to an oligonucleotide with a length of approximately 18 to 31 nucleotides that bonds to an area defined by nucleotides 123 to 168 of the sequence shown in SEQ ID NO:1. The oligonucleotide has in particular the sequence shown in SEQ 1D NO:3. The invention also relates to an oligonucleotide with a length of approximately 18 to 35 nucleotides that bonds to an area defined by nucleotides 60 to 300 of the sequence shown in SEQ 2D NO:1.

[0037] The invention additionally makes methods available for determining the presence of a SARS-associated virus or HPAC in which case the methods disclosed here are used. Furthermore, the invention makes

such methods available for detecting the presence of HPAC and also comprises the step of reporting said determination results.

[0038] The following examples are intended to describe the invention but not limit it.

EXAMPLES

Example 1

Isolation of viral RNA

[0039] The extraction of nucleic acids is carried out with the QIAamp Viral RNA-Mini-Kit (QIAGEN, 52904) or with the QIAamp Ultrasens Virus Kit (QIAGEN, 53704). The Ultrasens-Kit has the advantage that it makes possible the use of a 1000 µl specimen volume (instead of a 200 µl volume with the Mini-Kit). In both instances the nucleic acid is eluted in a volume of 60 µl. When using 5 µl eluate in a 20 µl PCR reaction a larger aliquot of the specimen (1/12 of 1000 corresponds to 83 µl specimen instead of 1/12 of 200 ml, that corresponds to a 17 ml specimen) can be analyzed for the presence of viral RNA.

LightCycler™ PCR

[0040] The amplification and detection of HPAC is carried out by real-time RT-PCR in a LightCycler device. The specificity of the detection

of PCR is based on the use of HPAC-specific primers and a fluorescent, double-marked oligonucleotide probe. The probe sequence is localized within the primer-defined HPAC amplicon.

[0041] This construction makes possible the use of fluorescence measurement for the direct, quantitative and specific detection of HPAC-specific DNA amplification within the closed PCR reaction vessel. The technology used in this step is based on the 5' exonuclease assay described in US patents 5,210,015 and 5,487,972. Bases and applications of this technology are readily obtainable from scientific publications, e.g., from Krupp et al., "Nucleic Acid Preparations of Pathogens from Biological Samples for Real-Time PCR Analysis", Nuclear Acids Isolation Methods (B. Bowien & P Dürre, editors), American Scientific Publishers, Stevenson Ranch, 2002.

Selection of the sequences of the primers and of the hybridization probe

[0042] Two criteria must be met. Completion: All known variants of HPAC must be detected. Specificity: all other closely related Coronaviridae must be excluded.

[0043] The following primers were selected based on a published, detailed sequence alignment (Drosten et al. (2003) "Identification of a Novel Coronavirus in Patients with Severe Acute Respiratory Syndrome) published in the New England Journal of Medicine at www.nejm.ora, April 10, 2003):

Sense 5' CCG	CGA AGA AGC TAT TCG TC 3'(SEQ ID NO:2); (20 nt; position 75-94)
Antisense 5' GTA	GGT TAG TAC CCA CAG CAT CTC TAG T 3' (SEQ 1D NO:3); (28 nt; complementary to position 139-166).

[0044] The sequence published in Drosten et al. was selected as probe sequence: Probe BNITMP 5' (6-FAM) TCG TGC GTG GAT TGG CTT TGA TGT (dabcyl) 3' (SEQ 2D NO:4); (24 nt; position 99-122).

[0045] The probe was marked on the 5' end with a reporter dye (here 6-FAM) and on the 3' end with a quencher dye (preferably a non-fluorescent quencher-like dabcyl). As is customary in the state of the art, the probe oligonucleotide is blocked on the 3' end in order to prevent enzymatic elongation. This elongation would result in a longer hybridization sequence that could cause a less specific hybridization with related, undesired sequences.

Experimental protocol for carrying out an HPAC detection with the aid of the LightCycler device

[0046] The following reaction conditions (or similar ones) can be used:

[See original table, p. 6, for alphanumeric data in this and all following tables. Only the German words are translated.]

Component	Volume (µl)
2x RT/Taq Reaction buffer	
HPAC-RNA or extracted nucleic acid from an unknown specimen	

Key: Sonde = probe

[0047] The following program was used in the LightCycler:

Program	Temperature [°C]	Time [sec]	Increase [°C/sec]	2nd target temperature	Step Size	Step Delay	Detection
Reverse transcription							
							none
Denaturing							
							none
Amplification (50 cycles)							
							none
							one
							none
Cooling off							
							none

Sensitivity of the HPAC detection

[0048] A PCR-derived construct was used that combined the HPAC amplicon sequence with the promoter sequence for the T7 RNA polymerase. A demonstration boundary of 120 HPAC genome equivalents per ml specimen was determined in a dilution series, which corresponds to 10 genome equivalents per 50 µl PCR reaction (see attached figures).

Kit components

[0049] All necessary materials, including positive controls (in-vitro transcript of HPAC RNA) and internal control JC (in-vitro transcript of a non-related sequence, e.g. a segment of the sequence of the lambda bacteriophage).

[0050] The master mix of the kit contains all required components including enzymes but no magnesium ions. Magnesium is contained in the kit as a separate solution. After the combination of both solutions of the kit with the isolated nucleic acid of the specimen, the mixture is used directly in the LightCycler device and the data analysis is possible without further treatment of the specimen.

[0051] The internal control RNA is contained in the kit as a separate solution. It can be selectively edited during the step of the specimen lysis already (in order to control the efficiency of the nucleic acid extraction). However, it can also be added later to the master mix (in order to exclusively control the efficiency of the RT-PCR).

Example 2

[0052] A specific protocol for the quantitative RT-PCR for detecting the SARS-associated virus is cited in the following.

[0053] RealArt™ HPA coronavirus LC RT-PCR reagents for use in the LightCycler device (Roche Diagnostics).

1. Contents

[0054]

	Marking and contents	... [illegible - ... reactions?]	... [illegible - ... reactions?]	... [illegible - ... reactions?]
Blue		2 x 12 react.		
Yellow				
Red				
Red				
Red				
Red				
...[illegible [Green?]				
White	Water (PCR quality"			

(QS quantification standard) (IC internal control)

2. Storage

[0055] The kit components should be stored at -20°. They are stable for 3 months at this temperature. Repeated defrosting and freezing (> 2x) should be prevented since this could result in a reduction of sensitivity. If the kit is to be used only periodically, the reagents should be frozen in aliquots. The storage at +4°C should not exceed a time period of 5 – 6 hours.

3. Additionally required materials and devices

- Powder-free disposable gloves
- RNA isolation kit (cf. 8.1 RNA isolation)
- Physiological saline solution (0.9% NaCl) containing 1 N acetyl cysteine
- Pipettes (adjustable to 1 – 20 µl)
- Sterile pipette tips with aerosol barrier
- Vortex mixer
- Table centrifuge with rotor for 2 ml reaction vessels
- LightCycler® capillaries, Roche Diagnostics
- LightCycler® cooling block, Roche Diagnostics
- LightCycler® device, Roche Diagnostics

4. General precautionary measures for the PCR

[0056] The user should always be aware of the following points:

- Pipette tips with filters should be used.
- The storage of positive material (specimens, controls and amplicons) should take place separately from all other reagents and their addition to the reaction mixtures should take place in a spatially separate apparatus.
- All components should be carefully defrosted at room temperature before the start of the assay.

- The defrosted components should be carefully mixed and centrifuged.

- The work should be performed speedily on ice or in a cooling block.

5. Information about the pathogens

[0057] Coronaviruses, a genus in the family of Coronaviridae, are large, encased RNA viruses with a positive strand that cause highly virulent diseases in humans and house pets. Two coronaviruses, of which it is known that they infect humans, cause one third of all diseases and are, in addition, a common cause of infections, associated with nursing care, of the upper respiratory tract in prematurely born babies.

[0058] It is assumed that a member of the Coronavirus family causes severe acute respiratory syndrome (SARS). The virus has not yet been classified. It is suggested in the literature that the virus be named human pneumonia associated coronavirus (HPAC). A part of a putative coronavirus polymerase gene was identified in a SARS patient by PCR by the Bernhard-Nocht Institute for Tropical Medicine in Hamburg as well as by cooperating laboratories. This assay was used to establish a commercially obtainable real-time RT-PCR system for the direct detection of this new type of coronavirus.

6. Principle of real-time PCR

[0059] The diagnoses of pathogens by polymerase chain reaction (PCR) is based on the amplification of specific areas of the genome of the pathogen. In real-time PCR the amplified product is demonstrated by fluorescent dyes. These dyes are customarily bonded to oligonucleotide probes that specifically bond to the amplified product. The following of the intensities of fluorescence during the course of the PCR (that is, in real time) makes the demonstration and the quantification of accumulating products possible without the reaction vessel having to be reopened after the course of the PCR.

7. Product description

[0060] The RealArt™ HPA coronavirus LC RT-PCR reagents is a ready-to-use system for the detection of HPA coronavirus RNA using a PCR in a LightCycler device (Roche Diagnostics). The HPA coronavirus LC master comprises reagents and enzymes for the specific amplification of an 80 bp area of the HPA coronavirus genome as well as for the direct demonstration of the specific amplicon in fluorimeter canal F1 of the LightCycler® device. In addition the RealArt™ HPA coronavirus LC RT-PCR reagents contains a second heterologous amplification system in order to identify a possible PCR inhibition. This is demonstrated as internal control (IC) in fluorimeter canal F3 and does not influence the analytic HPA

coronavirus RT-PCR. External positive controls (HPA coronavirus LC QS 1 – 4) are also made available that permit the determination of the pathogenic load. See Section 8.3 (quantification) for more information.

8. Protocol

8.1 RNA isolation

[0061] Numerous manufacturers offer kits for RNA isolation. The specimen volumes in the RNA isolation methods are dependent on the protocol used. The RNA isolation is carried out in accordance with the instructions of the manufacturer. The following isolation kits are recommended:

Nucleic acid isolation kit	Catalog number	Manufacturer
QIAamp UltraSens virus test kit (50)	53 704	QIAGEN
QIAamp viral RNA mini kit	52 904	QIAGEN

[0062] Attention. It is important to use native, provoked sputum (such as MTB). Mix the sputum specimen for 30 minutes with physiological saline solution (0.9% NaCl) containing 1% acetylcysteine. Pelletize the cells (approximately 600 µl) in a table centrifuge (10,000 g). Remove 140 µl of the supernatant and the corresponding cells parallel

(QIAamp viral RNA mini kit), add 560 µl AVL and proceed with the customary viral RNA protocol.

- The RealArt™ HPA coronavirus LC RT-PCR reagents should not be used in combination with isolation methods based on phenol.

- If the selected isolation kit does not contain any carrier DNA/RNA, it should be noted that the addition of carrier RNA (RNA homopolymer poly(A), Amersham Biosciences) at a concentration of 10 µg/ml lysis buffer to the specimen/lysis buffer mixing is recommended for the isolation of nucleic acid to a high degree.

- When using isolation protocols with ethanol-containing wash buffers, an additional centrifugation stage should be carried out before the elution in order to completely remove remaining ethanol. This prevents a possible inhibition of the PCR.

[0063] Important: The internal control of the RealArt™ HPA coronavirus LC RT-PCR reagents can be used directly in the isolation method (see 8.2 internal control).

8.2 Internal control

[0064] Internal control (HPA coronavirus LC IC) is made available. This gives the user the control of the isolation method as well as a check regarding a possible PCR inhibition. For this application the internal control of the isolation is added in a ratio of 0.1 µl per 1 µl elution volume. When

using the QIAamp viral RNA mini kit, for example, the RNA is eluted in 50 µl AE buffer. Accordingly, 5 µl of the internal control should initially be added. If the elution takes place, e.g., in 100 µl, a corresponding volume of 10 µl should be used. The amount of IC use depends only on the elution volume. The internal control should be added directly to the specimen/lysis buffer mixture. To the extent that an RNA isolation from a rather large number of specimens is necessary, the internal control can be added directly to the lysis buffer.

[0065] The IC can be selectively used exclusively for the checking of a possible PCR inhibition. For this use 05. µl of he IC and 3 µl HPA coronavirus LC Mg-Sol per text mixture are added directly to 12 µl HPA coronavirus LC master. For every PCR reaction 15 µl of the master mix are produced as described above 1 (The volumetric increase caused by the addition of the IC is not taken into account when producing the PCR assay. The sensitivity of the demonstration system is not adversely affected), and 5 µl of the purified specimen are subsequently added. If several specimens are produced for a PCR run, the volume of the HPA coronavirus LC master, of the HPA coronavirus LC Mg-sol and of the internal control is elevated in accordance with the number of specimens (see 8.4 Production of the PCR).

8.3 Quantification

[0066] The attached quantification standards (HPA coronavirus LC QS 1 – 4) are treated like the previously purified specimens and the same volume is used (5 µl). In order to produce a standard curve in the LightCycler® device all 4 quantification controls should be used and defined in the sample loading screen as standards with the specified concentrations (see LightCycler operating manual, version 3.5, chapter B, 2.4, Sample data entry). Furthermore, the standard curve produced as described can be used for subsequent runs, provided that at least one standard is used in the current run. To this end the previously produced standard curve must be imported (see LightCycler operating manual, version 3.5, chapter B, 4.2.5. Quantification with an external standard curve). However, this quantification method can result in deviating results on account of the variability between different PCR runs.

[0067] Attention: The quantification controls are defined as copies/µl in order to convert the determined values into copies/ml specimen material using the standard curve.

$$\text{Result (copies/ml)} = \frac{\text{result (copies/µl)} \times \text{elution volume (µl)}}{\text{specimen volume (ml)}}$$

8.4 Carrying out the PCR

[0068] It must be ensured that the cooling block as well as the capillary adapter (accessories of the LightCycler device were pre-cooled to

+4°C. The desired number of LightCycler capillaries is entered into the adapters of the cooling block. At least one quantification standard as well as one negative control (water, PCR quality) should be used per PCR run. In order to generate a standard curve all quantification standards made available (HPA coronavirus LC QS 1-4) should be used for each PCR run. Prior to each usage all reagents must be completely defrosted and mixed (by repeated piping up and down or by careful vortexing).

[0069] To the extent that the internal control is to be used in order to check not only a possible PCR inhibition but also the isolation method, the IC is added already to the isolation (see 8.2 Internal control). In this instance the following pipetting scheme should be used (see the schematic survey in figure 1):

	Specimen number	1	12
1· Production of the master mix			
	Total volume		
2. Production of the PCR assay	Master mix		15 µl each
	Specimen		5 µl each
	Total volume		20 µl each

To the extent that the IC is to be used exclusively in order to determine whether a PCR inhibition is present, it must be added directly to

the HPA coronavirus LC master. In this case the following pipetting scheme should be used (see the schematic survey in figure 2):

	Specimen number	1	12
1. Production of the master mix			
	Total volume		
2. Production of the PCR assay	Master mix		15 µl each
	Specimen		5 µl each
	Total volume		20 µl each

15 µl of the master mix are pipetted into the plastic reservoir of each capillary. Subsequently, 5 µl of the eluted specimen RNA are placed into each small tube. Accordingly, 5 µl of at least one of the quantification standards (HPA coronavirus LC QS 1-4) must be used as positive control and 5 µl water (PCR quality) as negative control. The capillaries are closed. In order to transfer the mixture out of the plastic reservoir of the capillary into the small glass tube the adapters containing the capillaries are centrifuged in a table centrifuge for 10 seconds at a maximum of 400 x g (2000 rpm).

8.5 Programming of the LightCycler® device

[0070] The LightCycler® PCR program for detecting HPA coronavirus RNA can be subdivided into four steps:

A: Reverse transcription of the RNA, figure 3,
B: Initial activation of the hot start enzyme, figure 4,
C: amplification of the cDNA, figure 5,
D: Cooling off, figure 6.

[0071] The LightCycler® device is programmed for these four steps in accordance with the parameters shown in figures 3-6. Particular attention should be paid to the adjustments for analysis mode, cycle program data and temperature targets. Further information about the programming of the LightCycler® device can be found in the LightCycler® user manual.

9. Data analysis

[0072] Interferences occur between the fluorimeter canals in multicolor analysis. The software of the LightCycler® device contains a file that is called the color compensation file and compensates these interferences. This file should be opened during or after the PCR run by activating the Choose CCC file button or the Choose CC data button. If no color compensation file is installed, the file should be generated in accordance with the instructions in the LightCycler® user manual. After the

color compensation file has been activated, separate signals for the analytical HPA coronavirus RT-PCR (F1/F2) and for the internal control (F3/back-F 11) should appear in fluorimeter canals F1 and F3 (When using older software versions (version 3.3 or older) the display function F3/back-F1 is not available. In this instance F3/F1 should be selected in order to display the internal control). In order to analyze quantitative runs, the instructions in Section 8.3 (quantification) should be followed.

[0073] The following results are possible:

1. A signal is detected in fluorimeter canal F1/F2. The result of the analysis is positive; the specimen contains HPA coronavirus RNA. In this case the detection of a signal in the F3/back-F1 canal can be dispensed with since high initial concentrations of HPA coronavirus RNA (positive signal in the F1/F2 canal) can result in a reduced or lacking fluorescence signal of the internal control in the F3/back-F1 canal (competition).

2. No signal is detected in fluorimeter canal F1/F2. At the same time a signal of the internal control appears in F3/back-F1 canal. The specimen contains no demonstrable HPA coronavirus RNA and can be evaluated as negative. In the case of a negative HPA coronavirus RT-PCR the detected signal of the IC excludes the possibility of a PCR inhibition.

3. No signal is detected in the F1/F2 or in the F3/back-F1 canal. No result can be determined. Information abaout possible error sourses and solutions can be found in Section 10 (troubleshooting).

[0074] Examples for positive and netative PCR reactions are shown in figures 7 and 8.

10. Troubleshooting

[0075] No signal with the positive controls (HPA coronavirus LC QS 1-4) in fluorimeter canal F1/F2:

- False programming of the LightCycler® device.
- Repeat the PCR with correct adjustments.

[0076] Weak or no signal in the internal control in fluorimeter canal F3/back-F1 and simultaneous lack of a signal in canal F1/F2.:

- The PCR conditions do not correspond to the protocol.
- Repeat the PCR with correct adjustments.
- The HPA coronavirus LC master was defrosted and frozen too often.
- The HPA coronavirus LC master was stored longer than 5-6 hours at +4°C.
- Observe the storage conditions cited in point 2 (storage). Repeat the PCR using a new HPA coronavirus LC master.
- The PCR was inhibited.
- Make sure that a recommended insulation method (see 8.1 RNA insulation) is used in strictly follow the instructions of the manufacturer.

[0077] Although the invention was described making reference to the above-cited examples, it should be understood that numerous modifications can be made without departing from the core of the invention. Accordingly, the invention is limited solely by the claims.

Sequence protocol

[See page 14 of the original for data and English words.]

< 110> Artus GmbH

120> Method for detecting a novel coronavirus associated with the severe acute respiratory syndrome (SARS).

<213> SARS-associated virus

<223> 6-FAM modification on the 5' end
dabcyl modification on the 3' end

Claims

1. A kit for detecting the virus associated with the severe acute respiratory syndrome (SARS-associated virus) called HPAC (Human Pneumonia-Associated Coronavirus) by real-time RT-PCR that comprises a forward primer with a length of approximately 18 to 31 nucleotides that bonds to an area defined by nucleotides 69 to 98 of the sequence shown in SEQ 1D NO:1 [sic – cf. 0031]; comprises a reverse primer with a length of approximately 18 to 31 nucleotides that bonds to an area defined by nucleotides 123 to 168 of the sequence shown in SEQ ID NO:1; and comprises a probe with a length of approximately 18 to 35 nucleotides that bonds to an area defined by nucleotides 89 to 132 of the sequence shown in SEQ ID NO:1, which probe is marked with two dyes, one of which is a fluorescent reporter dye and one is a quencher dye, and in which at least one dye is a fluorescent dye.

2. The kit according to Claim 1, characterized in that the forward primer has the sequence shown in SEQ ID NO: 2, the reverse primer the sequence shown in SEQ ID NO: 3 and the probe the sequence shown in SEQ ID NO: 4.

3. The kit according to Claim 1 or 2, characterized in that the reporter dye is FAM, 6-FAM, 5-FAM or ALEXA-288.

4. The kit according to Claims 1 to 3, characterized in that the quencher dye is TAMRA, DABCYL or QSY.

5. The kit according to Claims 1 to 4 that also contains enzymes and reagents required for carrying out a real-time RT-PCR reaction.

6. An oligonucleotide with a length of approximately 18 to 31 nucleotides that bonds to an area defined by nucleotides 69 to 98 of the sequence shown in SEQ ID NO:1.

7. The oligonucleotide according to Claim 6, that has the sequence shown in SEQ 1D NO:2.

8. An oligonucleotide with a length of approximately 18 to 31 nucleotides that bonds to an area defined by nucleotides 123 to 168 of the sequence shown in SEQ ID NO:1.

7. The oligonucleotide according to Claim 8, that has the sequence shown in SEQ 1D NO:3.

10. A kit for detecting the virus associated with the severe acute respiratory syndrome (SARS-associated virus) called HPAC (Human Pneumonia-Associated Coronavirus) by real-time RT-PCR that comprises a forward primer with a length of approximately 18 to 35 nucleotides that bonds to an area defined by the nucleotides from approximately 1 to approximately 240 of the sequence shown in SEQ 1D NO:1 ; comprises a reverse primer with a length of approximately 18 to 31 nucleotides that bonds to an area defined by the nucleotides from approximately 60 to approximately 300 of the sequence shown in SEQ ID NO:1; and comprises a probe with a length of approximately 12 to 40 nucleotides that bonds to an area defined by the nucleotides from approximately 21 to approximately 279

of the sequence shown in SEQ ID NO:1, which probe is marked with two dyes, one of which is a fluorescent reporter dye and one is a quencher dye, and in which at least one dye is a fluorescent dye.

11. The kit according to Claim 10, characterized in that the reporter dye is FAM, 6-FAM, 5-FAM or ALEXA-288.

12. The kit according to Claims 10, 11, characterized in that the quencher dye is TAMRA, DABCYL or QSY.

13. The kit according to Claims 10 to 12, that also contains enzymes and reagents required for carrying out a real-time RT-PCR reaction.

5 pages of drawings follow